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Attenuation of Anticonvulsant Effects of Diazepam After Chronic Treatment With Bicuculline

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SUZUKI, T., H. MOTEGI AND M. MISAWA. *Attenuation of anticonvulsant effects of diazepam after chronic treatment with bicuculline.* PHARMACOL BIOCHEM BEHAV 45(4) 881–887, 1993.—Changes in the GABAergic system after chronic treatment with bicuculline were examined in two strains of inbred rats, Fischer 344 (F344) and Lewis (LEW). Rats received an IP injection of either bicuculline (2 mg/kg) or vehicle once a day for 12 days. After this chronic treatment, the effects of diazepam (1 mg/kg, IP) and pentobarbital (20 mg/kg, IP) on bicuculline-induced convulsions were measured. Bicuculline was acutely infused into a tail vein at 0.0415 mg/min, and the infusion was terminated when rats showed seizure. Following the chronic bicuculline treatment, the anticonvulsant effect of diazepam, but not of pentobarbital, was significantly reduced as compared to its effect following chronic vehicle treatment in both strains. Both diazepam and pentobarbital showed a significant difference in anticonvulsant effects between strains (F344 > LEW). The hypnotic effects of muscimol, barbital, pentobarbital, and ethanol following chronic bicuculline treatment were examined. There was no significant difference in sleep time induced by these drugs between bicuculline- and vehicle-treated rats. These results suggest that the attenuation of diazepam's anticonvulsant effect after chronic bicuculline treatment may result from functional changes in benzodiazepine receptors and that the anticonvulsant effects of diazepam and pentobarbital may be influenced by genetic factors. Moreover, the hypnotic effects of several drugs tested are apparently not affected by chronic bicuculline treatment.

Bicuculline Diazepam Pentobarbital Anticonvulsant effect Hypnotic effect

THE GABA/benzodiazepine receptor complex appears to be a primary site for benzodiazepine action, and the pharmacological profile of the benzodiazepines could be explained by the facilitation of GABAergic transmission. It has also been thought that barbiturates and picrotoxin act on this complex (5,15,22); barbiturates augment the binding of GABA agonist (3,8,36) and of benzodiazepines to their respective receptors (11,12,17). GABA agonists enhance the binding of benzodiazepines, and this enhancement is blocked by bicuculline, a $GABA_A$ receptor antagonist (33). Conversely, GABA binding is also enhanced by benzodiazepines (7). The acute administration of bicuculline has been known to produce convulsions in rodents. In biochemical studies, there are some reports that changes in the GABAergic system were produced after chronic administration of bicuculline (9,23). However, in behavioral experiments there have been few reports examining the influence on behavioral effects of GABA agonist and benzodiazepines following chronic bicuculline treatment (19).

On the other hand, several pharmacogenetic studies have been conducted using inbred animals. There are many such studies on the effects of several drugs that act through the GABA/benzodiazepine complex, such as barbiturates (18,30, 32), alcohol (1,29,32), GABA, and benzodiazepines (31,35).

The purpose of the present study was to examine the changes in the GABAergic system on anticonvulsant and hypnotic effects after chronic treatment with the $GABA_A$ receptor antagonist bicuculline. We also investigated the influence of genetic factors on these effects.

METHOD

Animals

Two inbred strains of male rats, Fischer 344 (F344) and Lewis (LEW), were used in all experiments. At the beginning of experiment, F344 and LEW rats weighed 140–180 g and 180–220 g, respectively. These rats were housed in individual cages under a 12h L : 12h D cycle (light on 8:00 a.m.) and in a room maintained at constant temperature (22 ± 1°C) and humidity (55 ± 5%). Rats were allowed free access to rat chow pellets and tap water.

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Chronic Treatment with Bicuculline

Bicuculline (2 mg/kg, IP) was administered once a day at 2:00 p.m. for 12 days. Control animals were injected with a vehicle (saline plus 0.1 N HCl). The concentration of 0.1 N HCl in the vehicle was less than 5%. In acute studies, rats received the vehicle daily for 11 days and a single dose of bicuculline (2 mg/kg, IP) on day 12.

Anticonvulsant Effect

Following chronic treatment with bicuculline, anticonvulsant effects of diazepam and pentobarbital on bicuculline-induced convulsions were determined using an IV infusion method (20,21). At 24 h after the termination of bicuculline or vehicle treatment, diazepam (1 mg/kg) or saline was injected IP to bicuculline- or vehicle-treated rats, respectively. One-half hour later, bicuculline was infused into a tail vein at a rate of 0.83 ml/min using an infusion pump (Natsume Seisakusho Co., Tokyo, Japan). Bicuculline was dissolved in 0.1 N HCl and diluted in saline. The pH of the solution was adjusted to 3 using 0.1 N NaOH. The final concentration of bicuculline was 0.05 mg/ml. The onset time latency of seizure, such as continuous myoclonic jerking of neck and forelimbs, was measured. The seizure threshold was calculated using the latency to seizure appearance, drug concentration, infusion rate, and weight of the rat as follows:

$$\text{seizure threshold (mg/kg)} = \frac{\text{Bicuculline concentration} \times \text{Infusion rate} \times \text{Latency}}{\text{Body weight}}$$

In the experiments involving pentobarbital, pentobarbital was injected (20 mg/kg, IP) 20 min before the experiment, and its anticonvulsant effect was determined in the same way as in the diazepam experiment.

Hypnotic Effect

Hypnotic effects of 3 mg/kg muscimol, 200 mg/kg barbital, 40 mg/kg pentobarbital, and 3 g/kg ethanol were examined at 24 h after the termination of bicuculline or vehicle treatment. All of these drugs were administered IP. Upon losing the righting reflex, animals were placed on their backs in V-shaped troughs. The duration of loss of the righting reflex (sleep time) was recorded. Animals were judged to be awake when they could right themselves three times in 30 s.

Drugs

Bicuculline (Sigma Chemical Co., St. Louis, MO) was dissolved in saline by the addition of 0.1 N HCl. Diazepam (Profarmaco, Italy) was suspended in saline to which a drop of Tween-80 had been added. Muscimol (Sigma), sodium pentobarbital, and sodium barbital (Tokyo Kasei Industries, Co., Tokyo, Japan) were dissolved in saline. Ethanol (Wako Pure Chemical Industries, Co., Tokyo, Japan) was diluted in saline at 20 v/v%.

Statistical Analysis

The data were presented as the mean ± SEM. One-way repeated analysis of variance (ANOVA) followed by Newman-Keuls multiple-comparison test was used for statistical evaluation.

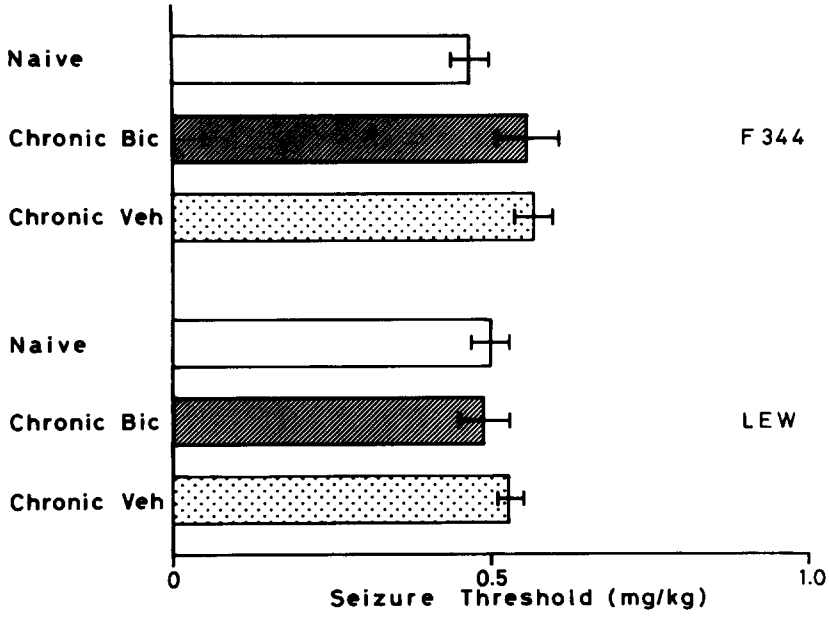

FIG. 1. Seizure thresholds to bicuculline (Bic) in two inbred strains of chronic bicuculline- and vehicle (Veh)-treated and naive rats, Fischer 344 (F344) and Lewis (LEW). Each column represents the mean with SE of five to seven animals.

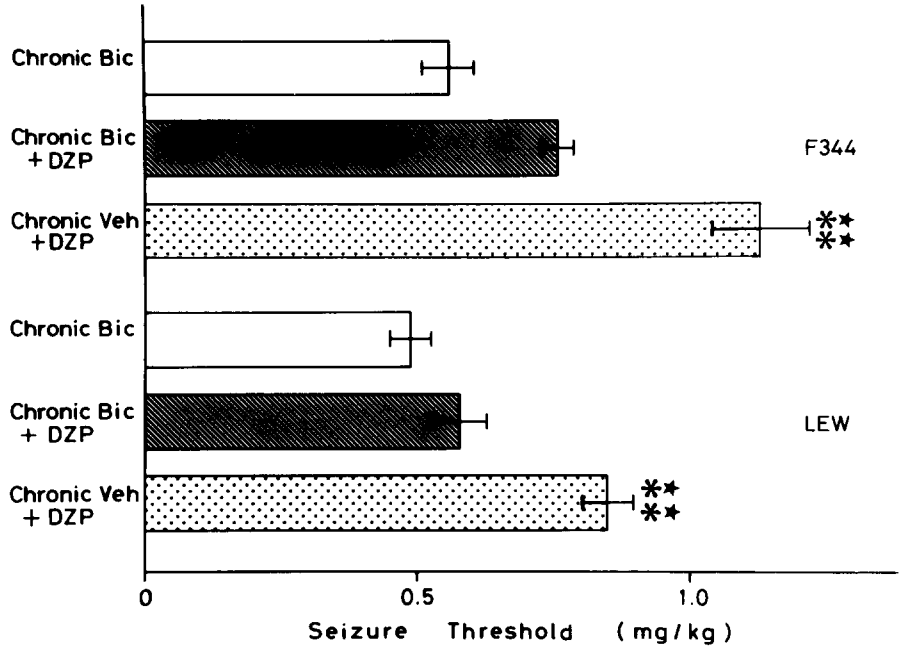

FIG. 2. Seizure thresholds to bicuculline (Bic) after administration of diazepam (DZP; 1 mg/kg, IP) in two inbred strains of rats, Fischer 344 (F344) and Lewis (LEW). Each column represents the mean with SE of five to six animals. $*p < 0.05$, $**p < 0.001$, compared to bicuculline alone group of respective strain. $**p < 0.01$ compared to diazepam group after chronic treatment with bicuculline.

RESULTS

Chronic Treatment with Bicuculline

There was no significant difference in the growth curve of body weights between bicuculline- and vehicle-treated rats. Changes in general behavior were not observed in either strain during chronic bicuculline treatment.

Anticonvulsant Effect

The seizure thresholds induced by acute bicuculline in chronic bicuculline- and vehicle-treated rats and in naive rats were 0.56 ± 0.05, 0.57 ± 0.03, and 0.47 ± 0.03 mg/kg in F344 rats and 0.49 ± 0.04, 0.53 ± 0.02, and 0.50 ± 0.03 mg/kg in LEW rats, respectively (Fig. 1). In neither strain, there were significant differences in seizure threshold to bicu-

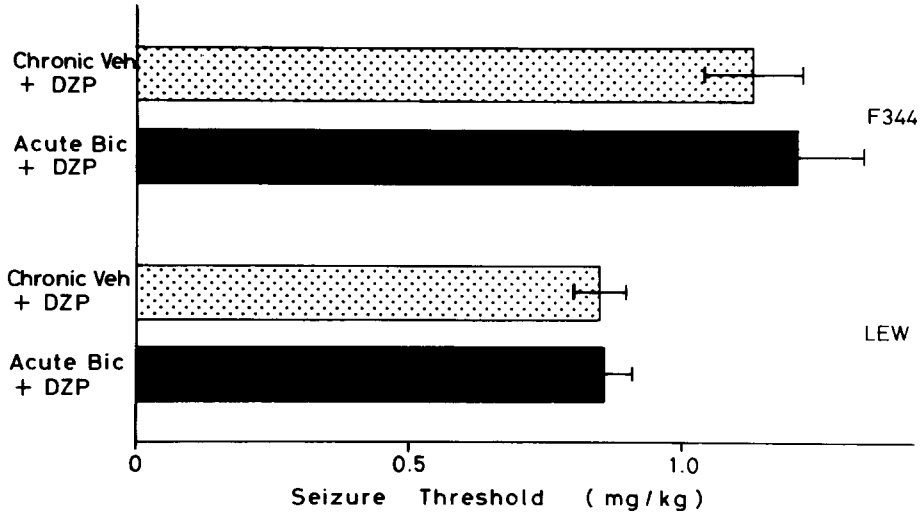

FIG. 3. Seizure thresholds to bicuculline (Bic) after administration of diazepam (DZP; 1 mg/kg, IP) following acute (single) bicuculline treatment in two inbred strains of rats, Fischer 344 (F344) and Lewis (LEW). Each column represents the mean with SE of five to six animals.

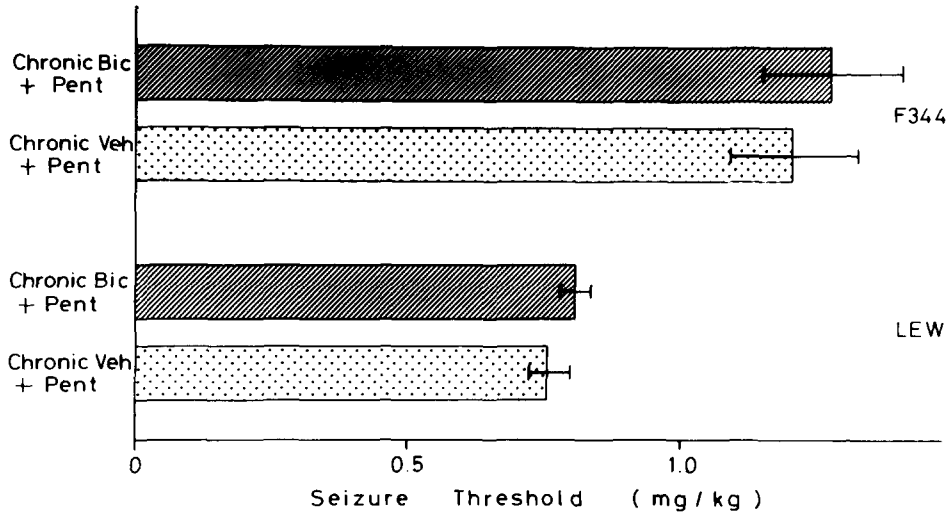

FIG. 4. Seizure thresholds to bicuculline (Bic) after administration of pentobarbital (Pent; 20 mg/kg, IP) in two inbred strains of chronic bicuculline- and vehicle (Veh)-treated rats, Fischer 344 (F344) and Lewis (LEW). Each column represents the mean with SE of five to six animals.

culline between chronic bicuculline- and vehicle-treated or naive rats. Diazepam significantly elevated the seizure threshold in the chronic vehicle-treated group ($p < 0.01$) but not the chronic bicuculline-treated group of F344 rats (Fig. 2). Further, the seizure threshold in chronic bicuculline-treated F344 rats was significantly lower than that of vehicle-treated F344 rats ($p < 0.01$). This attenuation of anticonvulsant effect of diazepam following chronic bicuculline treatment was also observed in LEW rats (Fig. 2). Further, the seizure thresholds following a single injection of bicuculline were 1.21 ± 0.12 for F344 and 0.87 ± 0.05 mg/kg for LEW rats. No significant differences were found between acute bicuculline- and vehicle-treated rats in the two strains (Fig. 3). However, as shown in Fig. 3, there was a significant difference in the anticonvulsant effect of diazepam between the two strains: The anticonvulsant effect of diazepam was more sensitive to an acute dose of bicuculline in F344 rats than in LEW rats ($p < 0.05$).

On the other hand, pentobarbital did not change the seizure threshold following chronic bicuculline treatment (Fig.

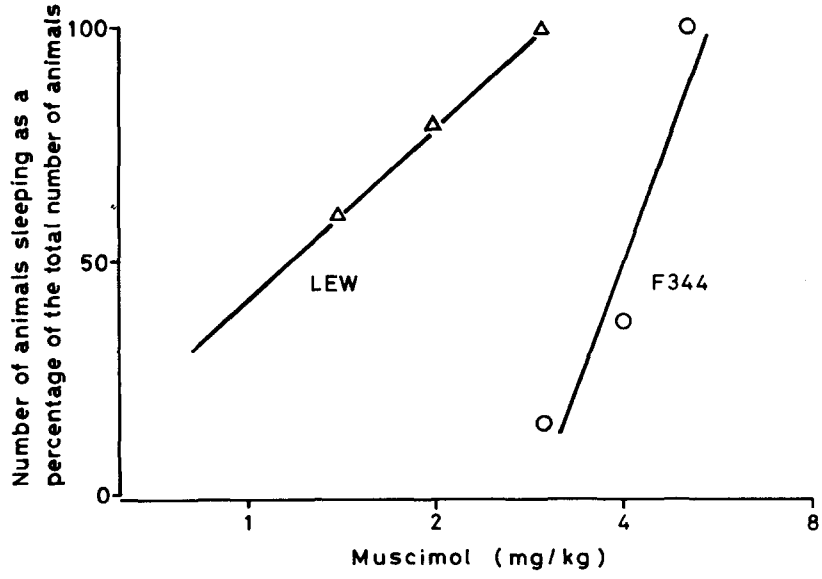

FIG. 5. Sensitivity to muscimol-induced sleeping in Fischer 344 (F344) and Lewis (LEW) rats. LEW rats were more sensitive to the hypnotic effect of muscimol than F344 rats by a factor of about 2.4.

TABLE 1

ONSET TIME AND SLEEP TIME OF SEVERAL DRUGS AFTER CHRONIC BICUCULLINE TREATMENT IN INBRED STRAINS OF RATS

Drug	Dose (mg/kg)	Strain	Pretreatment (min)	Onset Time (min)	Sleep Time (min)
Muscimol	3	LEW	Bicuculline	27.4 ± 0.5	148.7 ± 14.3
			Vehicle	30.3 ± 2.1	147.7 ± 9.9
Barbital	200	F344	Bicuculline	79.5 ± 15.6	203.7 ± 35.0
			Vehicle	93.5 ± 4.5	185.3 ± 32.8
		LEW	Bicuculline	56.2 ± 13.2	200.4 ± 15.8
			Vehicle	68.2 ± 15.2	200.8 ± 21.4
Pentobarbital	40	F344	Bicuculline	2.8 ± 0.1*	61.8 ± 5.4
			Vehicle	3.9 ± 0.2	65.8 ± 2.1
		LEW	Bicuculline	3.5 ± 0.3	59.1 ± 7.2
			Vehicle	3.1 ± 0.3	59.6 ± 5.7
Ethanol	3,000	F344	Bicuculline	2.5 ± 0.4	178.1 ± 11.8
			Vehicle	2.9 ± 0.4	159.0 ± 13.5
		LEW	Bicuculline	1.9 ± 0.2	158.8 ± 41.4
			Vehicle	2.4 ± 0.3	195.5 ± 30.2

F344, Fischer 344; LEW, Lewis.
*$p < 0.05$ compared to vehicle-treated rats.

4). The seizure thresholds of bicuculline after pentobarbital in chronic bicuculline- and vehicle-treated rats were as follows: 1.28 ± 0.13 and 1.21 ± 0.12 mg/kg for F344 rats and 0.81 ± 0.03 and 0.76 ± 0.04 mg/kg for LEW rats, respectively. There was a significant strain difference in the anticonvulsant effect of pentobarbital between the two strains (chronic bicuculline-treated groups: $p < 0.01$; chronic vehicle-treated groups: $p < 0.05$): The anticonvulsant effect of pentobarbital was more sensitive to an acute dose of bicuculline in F344 rats than in LEW rats.

Hypnotic Effect

There was no significant difference in muscimol-induced sleep time between bicuculline- and vehicle-treated LEW rats. However, muscimol-induced sleep time was not detected in F344 rats because this particular dose of muscimol (3 mg/kg) did not induce sleep in F344 rats. From the dose-response curve for muscimol, the potency ratio for hypnotic effect of muscimol in LEW rats was about 2.4 times greater than that in F344 rats (Fig. 5). The hypnotic effects of other drugs after chronic bicuculline treatment are shown in Table 1. There was no significant difference between bicuculline- and vehicle-treated rats except for the onset time of pentobarbital in F344 rats.

DISCUSSION

As mentioned above, chronic treatment with bicuculline, a $GABA_A$ receptor antagonist, produced attenuation of anticonvulsant effect of diazepam but not of pentobarbital. The doses of diazepam and pentobarbital used in this anticonvulsant experiment increased the seizure threshold approximately by a factor of two over the results seen in naive rats in our preliminary test.

Perez et al. (23) reported that chronic treatment with bicuculline increased GABA binding but decreased diazepam binding. Our results are consistent with their report in that the anticonvulsant effect of diazepam decreased following chronic bicuculline treatment. Benzodiazepines are generally believed to show their pharmacological effects through the enhancement of GABAergic transmission. In particular, the anticonvulsant effect of benzodiazepines appears to take place by directly enhancing GABA transmission. Further, it has been suggested that the pharmacological effects of benzodiazepines correlate with their receptor occupancy (4,24). The attenuation of the anticonvulsant effect of diazepam following chronic treatment with bicuculline observed in the present study may result from the decrease in benzodiazepine receptor sensitivity. However, Ito et al. (9) reported that $GABA_A$ receptors are upregulated after chronic administration of bicuculline but that there are no changes in benzodiazepine and picrotoxin binding sites. The cause of this discrepancy is not yet understood.

Another possible mechanism of the attenuation of diazepam's anticonvulsant effect, as suggested by several studies, may involve some additional effects of bicuculline that are not mediated by $GABA_A$ receptor blocking. Squires et al. (26) suggested that R 5135, a bicuculline-like GABA receptor antagonist, produced a conformational change in the [^{35}S]TBPS binding sites. It has been reported that bicuculline prevents the inhibition of TBPS binding by pentobarbital (34). Further, Liljequist and Tabakoff (13) indicated that bicuculline not only inhibits GABA-induced effects but also causes other effects that are probably not directly related to bicuculline's GABA receptor blocking. A variety of convulsant drugs have been shown to produce kindling, including pentylenetetrazol (6,25), picrotoxin (19), and FG 7142 (14,27). However, in the present study the attenuation of diazepam's anticonvulsant effect was apparently not due to kindling because the attenuation was only observed with diazepam and not with pentobarbital. Nutt et al. (19) also reported that although picrotoxin and pentylenetetrazol produced kindling to full seizures, bicu-

culline showed no tendency to increase susceptibility following chronic treatment with these drugs.

Low concentrations of barbiturates, including pentobarbital, potentiate $GABA_A$ receptor function (28). High concentrations of pentobarbital activate chloride channels directly (10). In the present study, we demonstrated that anticonvulsant effects of pentobarbital were not changed following chronic bicuculline treatment. These findings suggest that the action sites of barbiturates are scarcely influenced by chronic bicuculline treatment. However, Liljequist and Tabakoff (13) reported that the effect of pentobarbital on GABA receptors may be altered through an interaction of bicuculline in vitro. In addition, the anticonvulsant effect of barbiturates appears to be mediated through $GABA_A$ receptors (16). Bicuculline has been reported to reverse the anticonvulsant effect of pentobarbital but not that of phenobarbital (16). It is known that these two barbiturates differ in their pharmacological responses in mediating the GABA/benzodiazepine receptor complex (2).

More recently, there have been several studies of the genetic differences in pharmacological effects of, tolerance to, and dependence on barbiturates or benzodiazepines using selected lines and inbred animals. Wilks et al. (35) demonstrated that the number of specific benzodiazepine binding sites was higher in C3H/He than that in NIH mice, and these results may reflect strain differences in the GABA/benzodiazepine receptor complex. We also reported that F344 rats treated with barbital-admixed food for 36 days showed severe withdrawal signs compared to barbital-treated LEW rats (32) and that ethanol served as a strong positive reinforcer for LEW rats but a weak positive reinforcer for F344 rats (29). In the present study, we recognized clear differences in the anticonvulsant effects of diazepam and pentobarbital between F344 and LEW rat strains. Drug potencies were greater in F344 rats than in LEW rats. By contrast, the effects of the drugs on muscimol-induced sleep was greater in LEW rats than in F344 rats. If the pharmacological effects of benzodiazepines and barbiturates are mediated by the enhancement of GABAergic transmission, these opposite anticonvulsant and hypnotic effects between the different strains may reflect some differences in these enhancing mechanisms.

In conclusion, we found that chronic bicuculline treatment attenuated the anticonvulsant effect of diazepam but not of pentobarbital. However, hypnotic effects of both drugs were unchanged. These results suggest that the attenuation of anticonvulsant effect of diazepam may result from functional changes in benzodiazepine receptor sensitivity. On the other hand, the differences between strains in anticonvulsant effects of both diazepam and pentobarbital and in hypnotic effect of muscimol were clearly demonstrated, suggesting that genetic factors may strongly influence those effects in mediating the GABA/benzodiazepine receptor complex.

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